

# BIOCHEMICAL STUDIES OF ENVIRONMENTAL FACTORS ESSENTIAL IN TRANSFORMATION OF PNEUMOCOCCAL TYPES

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The phenomenon of transformation of pneumococcal types provides an outstanding example of the induction of specific and heritable modifications in microorganisms. Basically the phenomenon represents the transformation of a nonencapsulated (R) variant derived from one specific type of *Pneumococcus* into encapsulated (S) cells of heterologous specific type. By the technique employed at present, this is accomplished by growing the nonencapsulated cells in a special serum broth to which has been added the active fraction extracted from encapsulated pneumococci of a heterologous type. The production of a new polysaccharide capsule is induced in the R cells so that they acquire the type-specificity of the organisms from which the extract was obtained. The property of forming the new capsule is transmitted indefinitely to subsequent generations, and, in addition, the substance responsible for the induction of transformation is itself reduplicated in the transformed cells. It is thus apparent that one is dealing with hereditary bacterial modifications, which are predictable and subject to direct experimental control.

The chemical nature of the substance capable of bringing about this type of heritable change presents a problem of primary importance. Accumulated evidence based on the results of innumerable tests of the specificity and biological activity of various preparations, together with the data obtained by chemical, enzymatic, and serological analysis of the active material, has established beyond reasonable doubt that the active substance responsible for transformation is a specific nucleic acid of the desoxyribose type (2, 9, 10). These results suggest that nucleic acids in general may be endowed with biologically specific properties not hitherto demonstrable. Results confirming this view have been published recently in a preliminary report by Boivin and his co-workers on the role of desoxyribonucleic acid in inducing a transformation analogous to the pneumococcal phenomenon, using encapsulated strains of *E. coli* (3). These investigators state that evidence obtained by chemical and enzymatic techniques shows that desoxyribonucleic acid and not the protein of the nucleoprotein molecule is responsible for the specific effect. In so far as one can judge from this brief report, the results agree in principle with those obtained with *Pneumococcus*.

In the historical development of the problem of

transformation it is of interest to recall that Griffith (6), who originally described the phenomenon *in vivo*, was unable to obtain positive results *in vitro*. The first successful demonstration of the reaction in the test tube was carried out by Dawson and Sia (5) in nutrient broth containing anti-R rabbit serum. From that time on, serum or serous fluid of one sort or another has always been used and has been shown to be an essential factor, since in its absence it is impossible to induce transformation. The function of serum is not merely one of enrichment of the culture medium, however, since the nutrient broth itself contains adequate amounts of accessory growth factors required for initiation and maintenance of growth. It is evident, then, that serum provides essential environmental factors for pneumococcal transformation *in vitro*, and clarification of the role played by the serum is of foremost importance in understanding the nature of the phenomenon. The following questions naturally arise: Why is the presence of serum or serous fluid in the medium essential? Why are some sera capable of supporting transformation while others utterly fail? What components function as essential factors, how do they act, and what is the biochemical nature of their action in respect to the cellular changes evoked by the specific pneumococcal nucleic acid?

Although anti-R rabbit serum was used in the initial studies, in recent years this has been largely supplanted by human chest or abdominal fluids which occur as the result of a variety of pathological processes. These serous fluids invariably contain more or less R antibody, but they show marked variations in their ability to support transformation, which are unrelated to the antibody titer. It has been observed, for example, that chest fluids accumulating as the result of mechanical factors, as in the case of cardiac decompensation, regularly have little or no activity, while fluids formed as the result of tuberculous or acute infectious processes are usually highly effective. This suggests that one of the essential serum components may be present only in very low concentration in normal sera, but may increase in the course of infectious disease. This empirical observation has served as a useful guide in selection of effective sera.

The results of studies on the role of serum in the transforming reaction indicate that at least three essential constituents are involved. These are: (1) the R antibody, which causes agglutination of unencapsulated R pneumococci; (2) a dialyzable con-

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stituent; and (3) a protein factor, in addition to the R antibody. Each of the three components is considered individually in the following discussion. The evidence for assuming that the serum factor depends on the collective action of the three components is summarized, together with a description of certain experiments designed to elucidate the function of each and the mechanism of their combined effect.

#### THE ROLE OF R ANTIBODY

All sera that have proved effective in supporting transformation have contained R agglutinins, but despite this fact it was difficult to be certain that these antibodies were essential in the reaction. For example, there seems to be no relation between the titer of R antibody and the efficacy of the serum in the transforming system, and indeed some of the most potent sera have the lowest anti-R titers. The results of recent experiments, to be described below, provide some indication of the role of R antibody.

During growth in the serum broth used in the transformation system, the R cells agglutinate as they divide, so that each cell of the inoculum apparently gives rise to a colony, which becomes visible to the naked eye after several hours of growth (Fig. 1, a). Subsequently these colonial aggregates become larger and settle to the bottom of the tube, leaving a clear supernatant (Fig. 1, b).

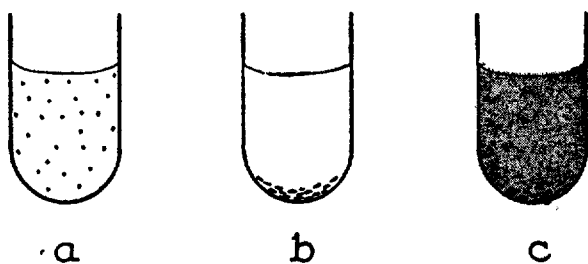


FIG. 1. Growth of pneumococci in serum broth. (a) Six-hour growth of R pneumococci. (b) Eighteen-hour growth of R pneumococci. (c) Transformation; 18-hour growth of R pneumococci in serum broth plus Type III transforming substance.

This latter fact is useful in the technique of the test, because newly formed S cells occurring as the result of transformation are not agglutinated by R antibody and grow diffusely throughout the tube (Fig. 1, c). Thus the appearance of the growth gives immediate presumptive evidence concerning the presence or absence of transformed pneumococci.

Colonial growth of R pneumococci somewhat analogous to that caused by anti-R results from the use of a semisolid medium (11). For example, in a viscous medium containing a low concentration of agar (0.2%) R pneumococci grow in loose aggre-

gates not unlike those formed by antibody agglutination. The growth differs, however, in that the colonies do not settle to the bottom of the tube (Fig. 2, a). S pneumococci give a fluffy, cotton-ball colony, which is readily distinguished from the R colony (Fig. 2, b), so that transformation in this type of semisolid medium can be recognized by the appearance of characteristic S outgrowths on the R colonies (Fig. 2, c). It has been found possible

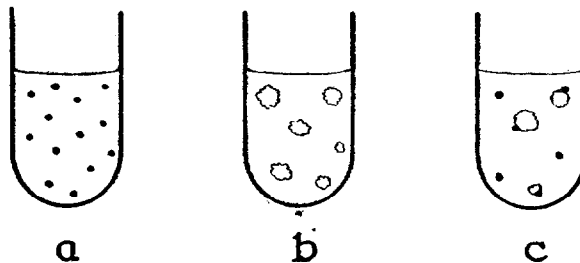


FIG. 2. Growth of pneumococci in semisolid medium. (a) Eighteen-hour growth of R pneumococci. (b) Eighteen-hour growth of S III pneumococci. (c) Transformation.

to bring about transformation in an agar semisolid medium containing normal rabbit serum but wholly lacking in R antibody. Neither normal rabbit serum nor the semisolid medium were by themselves capable of supporting transformation. This experiment suggests that the type of colonial growth produced by anti-R is an important factor and that when this type of growth is simulated by other means the anti-R can be dispensed with. It is important to note, however, that serum is required in the semisolid medium, although it is not essential that it contain R antibody.

Although it cannot be stated with certainty why colonial growth is required, it is possible that local reducing conditions arising in the aggregated cells are of primary importance. This thesis is supported by the results of experiments in which the medium is placed in a shallow layer not exceeding 1-2 mm. in depth. In the shallow layer, oxidizing conditions are promoted, and, even in the usual serum medium containing R antibody, manifest transformation does not occur. Attempts to reverse the effect of the shallow layer by the addition of reducing agents have not yielded consistent results, but on one occasion transformation was obtained in a group of flasks in which glutathione had been added to the usual serum medium. There is, then, some evidence that reducing conditions are essential in some phase of the transforming reaction.

On the basis of the evidence available at present it would appear that R antibody serves the purpose of causing an essential colonial aggregation of R pneumococci, which in turn results in local conditions, possibly reducing in character, that are required for transformation.

## DIALYZABLE SERUM CONSTITUENT

Early attempts at salt fractionation of serum factor by the classical methods of protein chemistry yielded totally inactive fractions. Some light has been thrown on these results by the discovery that a dialyzable constituent of the serum is essential. When an active serum is dialyzed against physiological saline, there is a progressive decrease in its efficacy in the transforming system, and if dialysis is sufficiently prolonged the serum becomes completely inactive. Under these conditions, however, the R antibody is unimpaired, and no denaturation of protein is apparent.

The period of dialysis required for complete inactivation varies for different sera from two days to three weeks. This suggests that the dialyzable substance may not be free in the serum but combined in loose linkage with a nondiffusible molecule. It is of interest, also, that sera are not inactivated by dialysis against distilled water, indicating that the salt ions may have some effect in displacing the dialyzable component from its linkage to the nondiffusible substance.

The problem of the nature of the dialyzable constituent has been approached by a study of the reactivation of serum rendered inactive by dialysis. Two distinct types of reactivation, which appear to be of different mechanism, have been devised. In the first place, if inorganic phosphate in concentrations of 0.005 M or above is added to dialyzed serum and the mixture incubated 1-2 hours, the

phate mixture after treatment, as indicated in Table 1, and the serum medium was then tubed in 2.0-cc. amounts for the test of ability to support transformation. Type III transforming substance was added and the tubes inoculated with a diluted culture of a susceptible R strain derived from *Pneumococcus* Type II. The tubes were incubated and the occurrence of transformation determined by the methods described in previous communications from this laboratory.

The data given in Table 1 demonstrate that reactivation of dialyzed serum can be achieved with inorganic phosphate if sufficient time is allowed for interaction. A relatively slow reaction occurs, which may conceivably be enzymatic in character. In contrast to this procedure, it is possible to bring about immediate reactivation of dialyzed serum by the addition of such materials as unheated Neopeptone or tryptic digest of casein. Further investigation of substances causing this type of reactivation disclosed that inorganic pyrophosphate has a similar effect and is not influenced by the presence of nutrient broth. The effects of pyrophosphate and Neopeptone, as compared with the effect of phosphate, are illustrated in Table 2.

Sodium pyrophosphate was added to dialyzed serum in a final concentration of M/150, Neopeptone sterilized by filtration in a final concentration of 0.2%, and inorganic phosphate as in the previous experiment. Nine volumes of nutrient broth were added immediately and the test for ability to

TABLE 1. REACTIVATION OF DIALYZED SERUM BY INCUBATION WITH PHOSPHATE

Source of Serum Factor	Additions	Period of Incubation before Addition of Nutrient Broth	Transforming Test with Type III Transforming Substance and Strain R36A Quadruplicate Tubes			
			R only*	R only	R only	R only
Dialyzed serum	None	2 hrs. at 37° C.	S III*	S III	S III	S III
Dialyzed serum	Phosphate	2 hrs. at 37° C.	R only	R only	R only	R only
Dialyzed serum	Phosphate	None	S III	S III	S III	S III
Whole undialyzed serum	None	None				

\* S III indicates the occurrence of transformation as evidenced by the recovery of encapsulated cells of *Pneumococcus* Type III, while the term "R only" means that transformation did not take place, and only unencapsulated R variants were recovered.

serum regains the ability to support transformation when added to broth in the usual concentration. The period of incubation of the serum with phosphate is essential. The interaction between phosphate and the serum appears to be prevented by the presence of nutrient broth, for if the latter is added at the same time as the phosphate, or after a short period of incubation, no reactivation is achieved. A protocol of an experiment demonstrating the reactivation of dialyzed serum by incubation with phosphate is given in Table 1.

The phosphate was added to dialyzed serum at a final concentration of M/150 in the form of  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer, pH 7.8. Nine volumes of broth were added to the serum or serum-phos-

support transformation carried out as before. The fact that pyrophosphate and Neopeptone restore the activity of dialyzed serum under conditions that are ineffective when simple inorganic phosphate is used is apparent from the data presented in Table 2. Transformation is as prompt in the reactivated serum medium as in the system containing undialyzed serum.

It is not known whether the effect of Neopeptone is dependent on the presence of inorganic pyrophosphate or whether other substances, such as certain organic phosphates, are perhaps also capable of reactivating dialyzed serum. A few organic phosphates, including adenosine triphosphate, pyridoxal phosphate, glycerophosphate, and fructose

diphosphate have been shown to be without effect.

The difference in the action of phosphate and pyrophosphate is emphasized in the case of globulin fractions of active sera. These fractions have for the most part been prepared by half saturation with ammonium sulfate followed by dialysis of the redissolved precipitate to remove excess sulfate ion. Globulin fractions so obtained are totally inactive in the transforming system, and even prolonged incubation with inorganic phosphate does not serve to reactivate them. On the other hand, immediate reactivation results from the addition of either in-

that some organ of the animal body contains the enzyme in much higher concentration than does serum and would serve as a more favorable source for possible purification and identification of the enzyme. To test this assumption, a preliminary survey was made of several rabbit organs by preparing simple saline extracts and testing them for the presence of the third component. The procedure used consisted of adding the extract to broth containing a small amount of concentrated rabbit R antibody plus unheated Neopeptone as a source of the dialyzable constituent. The broth containing these

TABLE 2. IMMEDIATE REACTIVATION OF DIALYZED SERUM BY PYROPHOSPHATE AND NEOPEPTONE

Source of Serum Factor	Additions	Treatment	Transforming Test with Type III Transforming Substance and Strain R36A Quadruplicate Tubes			
			R only*	R only	R only	R only
Dialyzed serum	None	Nutrient broth added immediately to all tubes.	R only	R only	R only	R only
Dialyzed serum	Phosphate		S III*	S III	S III	S III
Dialyzed serum	Pyrophosphate		S III	S III	S III	S III
Dialyzed serum	Neopeptone		S III	S III	S III	S III
Whole undialyzed serum	None		S III	S III	S III	S III

\* Symbols same as in Table 1.

organic pyrophosphate or Neopeptone. It appears that salt fractionation removes an unknown substance that is essential in the reaction by which phosphate effects reactivation but plays no part in the pyrophosphate reaction.

It seems inescapable that phosphate has an important function in the action of serum factor. Whether this effect is direct or indirect has not yet been conclusively determined. An example of an indirect action of phosphate in an enzymatic reaction has recently been provided by the work of Colowick and Price (4), who showed that phosphate brings about the reactivation of dialyzed preparations of muscle hexokinase. In this case, the phosphate is involved in a separate enzyme reaction which results in the release of guanine, an essential coenzyme for hexokinase activity.

#### THE THIRD COMPONENT OF SERUM

The activity of serum in supporting transformation does not depend solely on R antibody and the dialyzable component. This is demonstrated by the fact that purified R antibody is not effective in the transforming system even when fortified by the addition of pyrophosphate or Neopeptone to provide the dialyzable component. The third component of serum is a nondialyzable substance, which has not yet been sufficiently characterized to establish its chemical identity. However, the results of fractionation procedures indicate that it is protein in nature.

For the purpose of orienting further research, the possibility has been considered that the third factor may be an enzyme. If this is indeed the case, it seems highly probable on general grounds

added components was tested for its ability to support transformation. Positive results indicate that the organ extract has supplied the missing constituent, since as pointed out above, R antibody and dialyzable factor alone are unable to support transformation. Rabbit spleen proved to be a good source of the third component. To provide larger organs as source material, extracts of calf spleen and calf thymus were then tried, and it was found that thymus extracts were more active than those of any of the other organs tested. The apparent superiority of thymus extracts may be due in part to the almost complete absence of desoxyribonuclease, which inactivates the specific transforming substance and thus interferes with the test. Although these preliminary experiments have demonstrated that the third component is present in certain mammalian organs, little progress has been made in the isolation of this substance. Thymus extracts have proved to be exceedingly difficult to handle in fractionation attempts, because of the presence of mucoid material. Nevertheless, it is likely that a more favorable source will be found so that purified preparation of this component of the transformation system can be obtained and analyzed.

#### THE OCCURRENCE OF AN R VARIANT NOT REQUIRING DIALYZABLE COMPONENT FOR TRANSFORMATION

It has become increasingly apparent in recent years that bacterial populations, in common with populations of other living organisms, constantly undergo discontinuous variations, which must be presumed to be of the nature of genetic mutations. Inevitably, spontaneous variation of this sort be-

comes involved in a problem like that of pneumococcal transformation, in which relatively large bacterial populations are employed. The importance of mutation, or, as it is commonly called by microbiologists, dissociation, has been previously pointed out in connection with one aspect of this problem (2). The strain of *R. pneumococcus* (R36A) derived from Type II which is used in the majority of transformation experiments in this laboratory gives rise to numerous variants recognizable in a general way by slight alterations in colony topography. Certain of these variants acquire significance by virtue of the fact that they are totally insusceptible to the effects of the transforming substance. In all, four distinct variants of the parent R strain have been isolated which are not responsive to the influence of the transformation substance. Despite the fact that the R strain is cultivated continuously under more or less uniform conditions, subject to the limitations imposed by the complexity of the medium required for growth, the incidence of this type of dissociation is variable. This is presumably due to undefined differences in environmental conditions, which result in changes in the selective properties for a given mutant.

Another variant of strain R36A has been encountered, which is of special interest in connection with the dialyzable component of serum factor. This R variant, designated 6e, was originally isolated from a culture in a medium containing dialyzed serum, which appeared to be deficient for growth, since only a few of the R cells of the inoculum multiplied to form visible colonial aggregates. Strain 6e has been found to be not only susceptible in the transforming system, but completely independent of the presence of the dialyzable component of serum factor. Thus, it undergoes transformation readily in systems that contain dialyzed serum or globulin fractions of serum, without the addition of dialyzable component in the form of pyrophosphate or Neopeptone. Strain 6e therefore behaves in all respects as though it supplies its own dialyzable component or effects restoration of the serum by some analogous reaction. The protein factor is required, however, since transformation cannot be effected when only the R antibody is present in the system.

Strain 6e is of potential value in further study of the serum factors. Attempts have been made to determine in what way it differs from the parent strain with respect to synthesis or release of a substance replacing the dialyzable serum component. It has not been possible to show that larger amounts of such a substance accumulate either in the cells or in the supernatant medium of cultures of the variant.

#### EXPERIMENTS ON THE MECHANISM OF ACTION OF SERUM FACTOR

A series of experiments, which were designed to

provide a more intimate knowledge of the interaction between the specific transforming substance (pneumococcal desoxyribonucleic acid) and the susceptible pneumococcal cells, proved to have an important bearing on the problem of the role of serum factor. The customary procedure in demonstrating the phenomenon of transformation is to add the specific desoxyribonucleic acid to the serum medium and to inoculate with a susceptible strain of *R. pneumococcus*. Transformation becomes apparent after 16 to 20 hours' incubation, but little is known of the course of events during this period of incubation. The purified enzyme, desoxyribonuclease, which specifically inactivates the transforming substance (8, 9), has been used as a tool in an attempt to study certain phases of this problem.

TABLE 3. THE USE OF DESOXYRIBONUCLEASE IN DETERMINING THE TIME REQUIRED FOR THE UPTAKE OF TRANSFORMING SUBSTANCE BY SUSCEPTIBLE CELLS

Time of Addition of Enzyme after Inoculation	Transformation Test				
	Active enzyme; triplicate tubes			No enzyme; duplicate tubes	
1 hour	R only*	R only	R only	S III*	S III
2 hours	R only	R only	R only	S III	S III
3 hours	R only	R only	R only	S III	S III
4 hours	S III	S III	S III	S III	S III
5 hours	S III	S III	S III	S III	S III
6 hours	S III	S III	S III	S III	S III

\* Symbols same as in Table 1.

By adding desoxyribonuclease to the transforming system at various intervals after inoculation in a concentration known to cause almost immediate inactivation of the transforming substance, it is possible to determine the length of time required for the transforming substance to be taken up or "fixed" by the susceptible cells. An experiment of this type is illustrated in Table 3.

An amount of Type III transforming substance representing 1000 minimal effective doses was added to each of 30 tubes containing 2.0 cc. of serum medium. The tubes were all inoculated with 0.05 cc. of a suitable dilution (containing approximately 1000 cells) of the susceptible R strain, R36A, and then incubated at 37° C. At hourly intervals groups of five tubes were treated in the following way. To three of the tubes were added 4.0 µg. of purified desoxyribonuclease in 0.2 cc. of nutrient broth containing 0.03 M MgSO<sub>4</sub>; and to the remaining two tubes was added 0.2 cc. of the MgSO<sub>4</sub> broth without enzyme, as control on the effect of the agitation required to mix the enzyme thoroughly with the culture. The cultures were incubated overnight and the presence of transformation determined by

the usual procedure. The results are recorded in Table 3.

It will be seen that the addition of desoxyribonuclease at any time up to four hours after inoculation interferes with the reaction so that transformation does not occur, and it is therefore likely that throughout this period the transforming substance is readily accessible to the action of the enzyme. After four hours, on the other hand, the addition of desoxyribonuclease has no observable effect on the course of the reaction. This type of experiment was repeated with essentially the same results on several occasions and with different sera. Consequently, it appears that growth of the R cells in serum medium for 3 to 5 hours is required before the specific desoxyribonucleic acid is taken up by the cells and thus protected from enzymatic destruction. Further experiments have demonstrated that this does not depend solely on the increase in population (from the original inoculum of 1000 cells to an approximate 1,000,000 cells at 4 hours) and the consequent appearance of susceptible variants.

Confirmation of the importance of the four-hour period of growth is provided by experiments in which the R cells are grown in serum medium in the absence of the specific transforming substance. After 4 to 5 hours' growth under these conditions the cells are so "sensitized" that, when they are transferred to a medium containing the transforming substance, the latter is taken up in as short a time as 15 minutes. That is, desoxyribonuclease has no effect on the outcome of the transforming test when added 15 minutes after the previously "sensitized" cells are brought into the presence of the specific desoxyribonucleic acid. If the transfer is made after shorter periods of growth in the serum medium—e.g., 2 to 3 hours—the "sensitization" has apparently not taken place and rapid fixation of the transforming substance cannot be demonstrated. Furthermore, if growth in the serum medium is prolonged 7-8 hours or more the "sensitization" is lost, indicating that the alteration in the cells is not permanent but is affected by further environmental changes that occur on continued growth. These experiments demonstrate that the events which occur in the first four hours of growth of R cells in the transforming system are independent of the presence of the specific transforming substance. It must be concluded that growth under these conditions alters the cell in some way, or provides suitable environmental conditions, so that interaction between the cell and transforming substance can take place.

The relation of these experiments to the role of serum factor becomes apparent from the fact that a complete and active serum containing all the essential components must be present in order to achieve "sensitization" of the cells during the four-hour period. Growth in the presence of purified R antibody, in serum inactivated by dialysis, or in

globulin fractions of active sera, fails in each case to "sensitize" the cells. Thus the hypothesis is suggested that the major part played by serum in the transforming system is concerned with a modification of the R cell so that the specific transforming substance can be taken up.

#### DISCUSSION

It seems well established that the environmental conditions required for transformation of pneumococcal types *in vitro* depend on the combined action of several distinct factors. The present studies fall far short of the ultimate goal of determining the chemical nature and mode of action of the various factors involved. However, they provide a basis for and give direction to further research and make possible the formulation of a tentative hypothesis concerning the role played by serum in the transforming reaction.

The experiments in which desoxyribonuclease was used to inactivate the transforming substance free in the reaction system have demonstrated that the specific desoxyribonucleic acid does not participate in the preliminary phase of "sensitization" which takes place during the first four hours. On the other hand, the three known components provided by serum or serous fluid are all required during this four-hour period. At present, the most reasonable interpretation of the available data is that the action of the serum factors during this early phase results in alterations at the surface of the R cells so that they are capable of taking up or adsorbing the specific transforming substance. The alternative interpretation that the serum provides a strongly selective environment for growth of R mutants susceptible to transformation has been discarded on the basis of several important considerations. First, "sensitization" takes place in a relatively short time and is not dependent to any marked degree on the size of the original inoculum or on the total population. During the last 30 minutes of the four-hour period the number of "sensitized" cells increases from zero to at least 5000, representing approximately 0.5% of the total population. The final and most important evidence against the assumption that selection of a mutant is involved depends on the fact that the state of "sensitization" is temporary and readily lost. Cells that have been "sensitized" by growth for 4 hours in serum medium can be deprived of their acquired "sensitization" by repeated washing with nutrient broth or simply by allowing them to grow an additional 2 to 4 hours in the serum medium.

If it is assumed, then, that the action of the environmental factors is exerted at the surface of the R cells, the problem resolves itself into one of determining the nature of the alteration of the cell surface and the way in which the serum components bring it about. Perhaps the most attractive hypothesis is that the reaction is enzymatic in char-

acter and is dependent upon the action of an unidentified enzyme, represented by the third component of the serum, on certain specific groupings at the cell surface. It is well established that profound but temporary alterations in the peripheral mosaic of living bacterial cells can be brought about by enzymatic action. For example, the bacterial enzyme that hydrolyzes the capsular polysaccharide of *Pneumococcus* Type III is capable of removing the capsule from viable Type III organisms (1). The functions of capsule formation and polysaccharide synthesis are not impaired, however, and in the absence of the active enzyme, descendants of cells so treated again have all the characteristics of normal Type III cells. A similar phenomenon has been described in the case of hemolytic streptococci, from which one of the surface antigens, the M protein, can be removed by the use of proteolytic enzymes without affecting the viability of the bacteria (7). Therefore, in the case of the transforming reaction, it is not unreasonable to suppose that less extensive reversible alterations at specific sites of the surface of pneumococcal cells can result from enzymatic action, and that these alterations make possible the adsorption or penetration of the specific desoxyribonucleic acid.

In terms of the enzyme hypothesis, the occurrence of a dialyzable constituent is referable to a dissociable cofactor. The reactivation of dialyzed serum by incubation with phosphate has its counterpart in the work of Colowick and Price (4) in which the hexokinase activity of dialyzed muscle extracts is restored by incubation with phosphate. In this latter case, the release of a cofactor, guanine, by a secondary enzymatic reaction involving ribonucleic acid is responsible for reactivation. However, in the restoration of the transforming activity of dialyzed serum the immediate effect of pyrophosphate suggests that a different type of reaction is involved than in the case of hexokinase.

The function of the essential colonial aggregation of the R cells resulting from the action of R antibody is more difficult to interpret from the point of view of the enzyme hypothesis. However, bacterial aggregation may have an important relation to the period of growth required for "sensitization" of the cells, since at four hours the individual aggregates are just beginning to reach a size (about 1000 cells) which could provide special local conditions. It is conceivable that these local environmental changes, reducing or otherwise, provide essential conditions for the action of the enzyme. Thus, it is not necessary to make the unlikely assumption that the preliminary four-hour period is required because of the slow action of the hypothetical enzyme.

It scarcely need be reiterated that these considerations are tentative in character and subject to modification in the light of future results. How-

ever, the various assumptions discussed above provide the basis for a useful working hypothesis, the validity of which can be further tested experimentally. The results of the present studies leave little doubt that the contribution of serum to the transformation system is complex and dependent on at least three components: the R antibody, an additional protein component, and a dialyzable factor which may be combined with the protein component as it occurs naturally. Furthermore, evidence is presented which strongly suggests that the role of serum is concerned with alteration of the surface of the R cells so that the specific desoxyribonucleic acid is taken up or adsorbed.

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